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(57) Abstract

A replication-defective retroviral vector carrying a cytochrome P450 gene under transcriptional control of target cell specific regulatory elements or promoters, or X-ray inducible promoters.

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Cytochrome P450 transducing retroviral vectors

The present invention relates to replication-defective retroviral vectors encoding a cytochrome P450 gene which are capable of converting harmless cancer prodrugs into cytotoxic metabolites. Further, the present invention relates to the use of such retroviral vectors for the preparation of medicaments for the treatment of various cancers, or ablation of tumours.

Background of the Invention

The anti-cancer drugs used to treat tumours are in most cases applied systemically and spread through the whole body of the patient. The high systemic dose of such drugs required for cancer treatment is combined with unpleasant side-effects for the patient.

In an attempt to circumvent this problem, cancer-prodrugs that have to be metabolised or activated in the body before they become cytotoxic have been used. Unfortunately, human tumours that contain appropriate high levels of the activating enzymes are rare. The main site for activation of prodrugs is the liver and to ensure that a tumour, at a distant site, receives a sufficient dose of the activated drug, the amount of activated prodrug produced in the liver has to be quite high and again this leads to toxic side effects for the patient.

One strategy by which these problems of high systemic concentration of activated drugs could be circumvented would be to provide means for activation of the prodrug directly in or near the site of the tumour. This strategy would require that tumour cells, or cells at the site of a tumour are genetically transformed to produce high amounts of the enzymes required for metabolising the cancer prodrugs. Retroviral vectors are ideally suited for the stable delivery of genes to cells since the retrovirus is able to integrate the DNA form of its genome into the genome of the host cell and thus all daughter cells of an infected cell will carry the retroviral vector carrying the therapeutic gene. A further advantage is that most retroviruses only infect dividing cells and they are therefore ideal gene delivery vehicles for tumour cells.

A variety of cytotoxic genes carried by retroviral vectors have already been tested. These genes encode enzymes which convert substances that are pharmacodynamically and toxicologically inert even at high dose-levels but which can be converted *in vivo* to highly active metabolites (Connors, T. A. (1995). Gene Therapy 2: 702-709).

In cancer chemotherapy appropriately designed prodrugs have been found to be effective in the treatment of animal tumors possessing high levels of an activating enzyme (Connors, T. and Whisson, M. (1966), Nature 210: 866 867 and Cobb, L. et al (1969), Biochemical Pharmacology 18: 1519-1527). Clinical results were, however, disappointing since it was found that human cancers that contained appropriately high levels of activating enzymes were rare (Connors, T. (1986), Xenobiotica 16: 975-988). Virally directed enzyme prodrug therapy (VDEPT) and the more general gene directed enzyme prodrug therapy (GDEPT) are related in that they also aim to destroy tumour cells by the tumour specific activation of a prodrug. However, in this case, the gene encoding the enzyme is either specifically targeted to malignant cells or is under the control of a specific promoter.

Up to now most of the efforts directed towards prodrug therapy have concentrated on the use of the human Herpes Simplex Virus thymidine kinase (HSV-tk) as a suicide gene. Although the HSV-tk enzyme in combination with the prodrug ganciclovir (GCV) has been recommended as a good system for GDEPT (Culver, K. et al., (1992), Science 256: 1550-1552, Ram, Z. et al., (1993), Cancer Research 53: 83-88 and Chen, S., Shine, H. et al., (1994), Proc. Natl. Acad. Sci. 91: 3054-3057) there are a number of theoretical considerations that would suggest that it is by no means the best combination. First, it is an S-phase specific agent with no effect on resting cells. This is because the GCV monophosphate is short lived and has to be present when cells are entering the S-phase to give a toxic effect. The HSV-tk phosphorylates GCV to the monophosphate form (a reaction that cannot be performed by mammalian enzymes) which is then phosphorylated by cellular enzymes to the triphosphate form and incorporated into DNA. Second, the active drug is a triphosphate and would not be expected to diffuse freely to cause a bystander effect. However a bystander effect has been observed both *in vitro* and *in vivo* although metabolic cooperation appears to be involved and in the latter case some of the effect may be an indirect one involving an immune component (Bi, W. , Parysek, L. et al., (1993), Human Gene Therapy 4: 725-731, Vile, R. and Hart, I. (1993), Cancer Research 53: 3860-3864 and Freeman, S., Abboud, C. et al., (1993), Cancer Research 53: 5274-5283). One disadvantage is that the bystander effect is dependent on a cell-cell contact. This may be due to the presence of gap-junctions formed by intimate contact between the transduced and the surrounding cells which enable the transfer of phosphorylated ganciclovir.

Recently, interesting results have been reported with cells that have been transfected with the gene encoding the rat cytochrome P450 form 2B1 and then treated with cyclophosphamide (Chen, S., Shine, H et al., (1994), Proc. Natl. Acad. Sci. 91: 3054-3057).

Objects of the Invention

It is an object of the present invention to provide novel replication-defective retroviral vectors carrying a gene encoding cytochrome P450 under transcriptional control of a target cell specific regulatory element or promoter, or an X-ray inducible promoter, which can be transfected into a packaging cell lines for the producing recombinant retroviral particles useful for gene therapy of various cancers.

Summary of the Invention

The present invention then, inter alia, comprises the following, alone or in combination:

A replication-defective retroviral vector carrying a cytochrome P450 gene under transcriptional control of a target cell specific regulatory element or promoter, or an X-ray inducible promoter;

a replication-defective retroviral vector as above, wherein the vector comprises a 5' LTR region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence containing a target cell specific regulatory element or promoter, or an X-ray inducible promoter, followed by the R and U5 region, characterized in that at least one of the coding sequences codes for cytochrome P450;

a replication-defective retroviral vector as above, wherein the target cell specific regulatory element or promoter is selected from one or more elements of the group consisting of WAP, MMTV, b-lactoglobulin and casein specific regulatory elements and promoters; pancreas specific regulatory elements and promoters including carbonic anhydrase II and b-glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic specific regulatory elements and promoters and MMTV specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland;

a replication-defective retroviral vector as above, wherein said LTR regions are selected from at least one element of the group consisting of LTR's of MLV, MMTV, MSV, SIV, HIV, HTLV, FIV, FeLV, BLV and MPMV;

a replication-defective retroviral vector as above, wherein said retroviral vector is based on a BAG vector, or a pLXSN vector;

a replication-defective retroviral vector as above, wherein said retroviral vector is pLX2B1 prepared as described in example 1;

a replication-defective retroviral vector as above, wherein said retroviral vector is pc3/2B1 prepared as described in example 2;

a replication-defective retroviral vector as above, wherein the retroviral sequences involved in integration of the retrovirus are altered or at least partially deleted;

a replication-defective retroviral vector as above, wherein said regulatory elements or promoters are regulatable by transacting molecules;

a packaging cell line transfected with a replication-defective retroviral vector as above; said packaging cell line harbouring at least one retroviral or recombinant retroviral construct coding for the proteins required for said retroviral vector to be packaged;

a packaging cell line as above wherein the packaging cell line is of rodent, canine, feline or human origin, and is histocompatible with human tissue;

a packaging cell line as above, wherein the packaging cell line is selected from the group consisting of psi-2, psi-Crypt, psi-AM, GP+E-86, PA317 and GP+envAM-12;

a recombinant retroviral particle produced by culturing a packaging cell line as any above under suitable conditions optionally followed by isolation of the recombinant retroviral particle produced;

a pharmaceutical composition comprising a recombinant retroviral particle as above, or a packaging cell line as above;

a packaging cell line as above encapsulated in a porous membrane which is permeable to the recombinant retroviral particles produced by said packaging cell line;

a packaging cell line as above which is encapsulated in a complex formed from cellulose sulphate and polydimethyldiallylammonium;

a method for the ablation of tumour cells comprising administering to a subject in need thereof, a therapeutically effective amount of a recombinant retroviral particle as above, a packaging cell line as above, or an encapsulated packaging cell line as above and, either simultaneously or with a time span, a cancer prodrug which can be activated by cytochrome P450.

a method as above wherein the tumour cells are cells of a breast tumour, or a pancreatic tumour;

a method as above wherein the recombinant retroviral particle, the packaging cell line, or the encapsulated packaging cell line is administered by injection, or by implantation into the tumour, or the site of the tumour;

the use of a recombinant retroviral particle as above, a packaging cell line as above, or encapsulated packaging cell line as above for the preparation of a pharmaceutical composition useful for the ablation of tumour cells;

a retroviral provirus integrated in the human genome carrying a cytochrome P450 gene under transcriptional control of a target cell specific regulatory element or promoter, or an X-ray inducible promoter; and

a human cell containing a cytochrome P450 gene under transcriptional control of a target cell specific regulatory element or promoter, or an X-ray inducible promoter.

Detailed description of the Invention

Cytochrome P450's form a broad group of mono-oxygenases that catalyze oxidation of a wide range of substrates. They are produced by some bacteria, yeasts, and by higher organisms, where they play a role in detoxification of xenobiotics, bioactivation reactions, and metabolism of various endogenous compounds.

Cytochrome P450 catalyses the hydroxylation of the commonly used cancer prodrugs cyclophosphamide (CPA) and ifosfamide to their active toxic forms. Normally the expression of

the patient's endogenous cytochrome P450 gene is limited to the liver, and anti-tumor effects of systemically applied CPAs depend upon the subsequent systemic distribution of toxic drug metabolites from the liver. This has led to toxicity problems since the activated drug not only affects the tumor but also affects other normal patient tissues such as bone marrow and kidney.

A therapeutic approach, where the cytochrome P450 gene is selectively introduced directly into tumour cells, and overexpressed in these cells, would circumvent this problem. Toxic metabolites produced from the transduced tumour cells affect surrounding non-transduced tumor cells in a concentration gradient dependent manner. An additional advantage of the cytochrome P-450/CPA system is the lack of dependency upon cell replication for cytotoxic effects on the surrounding cells. This is because one of the active metabolites generated causes interstrand crosslinks regardless of the cell cycle phase. Later on, during DNA synthesis, these interstrand crosslinks result in cell death.

Retroviral vectors are the most commonly used gene transfer vehicles for the clinical trials that have been undertaken to date. Most of these trials have, however, taken an *ex vivo* approach where the patient's cells have been isolated, modified in culture and then reintroduced into the patient.

For the treatment of cancers, it would be feasible to isolate cells from a patient (either tumour cells or normal cells) infect them *in vitro* with a recombinant retroviral particle carrying the a gene encoding cytochrome P450, and then return them to the patient in the vicinity of the tumor. This approach, however, is extremely labour intensive because each patient cells must be isolated, cultured, transduced with the gene construct and successfully returned without infection by adventitious agents. The cost and time involved in such an approach limits its practical usefulness.

Alternatively an allogenic approach could be envisaged, where one type of cells are infected with a recombinant retroviral particle carrying a gene encoding P450, and then used for therapy of many different patients. Such an approach is much more feasible, assuming that problems of immune rejection can be overcome. Most tumours, however, are not suitable for *ex vivo* gene therapy.

Ideally, the gene encoding cytochrome P450 should be introduced *in vivo* into the tumour cells, or into cells in the vicinity of the tumour.

The delivery of genes *in vivo* introduces a variety of new problems. First of all, and above all, safety considerations have to be addressed.

A major concern for eventual *in vivo* gene therapy, both from a safety stand point and from a purely practical stand point, is the targeting of the expression. It is clear that therapeutic genes carried by vectors should not be indiscriminately expressed in all tissues and cells, but rather only in the requisite target cell. This is especially important when the genes to be transferred are such prodrug activating genes designed to ablate specific tumour cells. Ablation of other, non-target cells would obviously be very undesirable.

The essentially random integration of the proviral form of the retroviral genome into the genome of infected cells has posed a serious ethical problem because such random integration may lead to activation of proto-oncogenes and thus lead to the development of a new cancer. Most researchers would agree that the probability of a replication defective retrovirus, such as all those currently used, integrating into or near a cellular gene involving in controlling cell proliferation is vanishingly small. However, it is generally also assumed that the explosive expansion of a population of replication competent retroviruses from a single infection event, will eventually provide enough integration events to make such a phenotypic integration a very real possibility.

Retroviral vector systems are optimized to minimize the chance of replication competent virus being present. It has however, been well documented that recombination events between components of the retroviral vector system can lead to the generation of potentially pathogenic replication competent virus and a number of generations of vector systems have been constructed to minimize this risk of recombination (Salmons, B. and Günzburg, W. H. (1993), Human Gene Therapy 4(2): 129-41.

Retroviral vector systems consist of two components:

- 1) The retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins have been replaced by therapeutic genes. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2) A cell line that produces large quantities of the viral proteins, however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with one or more plasmids carrying the genes enabling the modified retroviral vector to be packaged.

To generate a recombinant retroviral particle, the retroviral vector is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic gene is transcribed from the retroviral vector and packaged into the modified retroviral particles. These recombinant retroviral particles is then used to infect tumour cells during which the vector genome and any cytotoxic gene becomes integrated into the target cell's DNA. A cell infected with such a recombinant viral particle cannot produce new vector virus since no viral proteins are present in these cells but the DNA of the vector carrying the therapeutic is integrated in the cell's DNA and can now be expressed in the infected cell.

A number of retroviral vector systems have been previously described that should allow targeting of the carried cytotoxic genes (Salmons, B. and Günzburg, W.H. (1993), Human Gene Therapy 4(2): 129-41). Most of these approaches involve either limiting the infection event to predefined cell types or using heterologous promoters to direct expression of linked heterologous therapeutic genes to specific tumor cell types. Heterologous promoters are used which should drive expression of linked genes only in the cell type in which this promoter is normally active or/and additionally controllable. These promoters have previously been inserted, in combination with the therapeutic gene, in the body of the retroviral vectors, in place of the *gag*, *pol* or *env* genes.

The retroviral Long Terminal Repeat (LTR) flanking these genes carries the retroviral promoter, which is generally non-specific in that it can drive expression in many different cell types (Majors, J. (1990), in "Retroviruses - Strategies of replication (Swanstrom, R. and Vogt, P. K., Eds.): Springer-Verlag, Berlin: 49-92). Promoter interference between the LTR promoter, and heterologous internal promoters, such as the tissue specific promoters, described above, has been reported. Additionally, it is known that retroviral LTR's harbor strong enhancers that can, either independently, or in conjunction with the retroviral promoter, influence expression of cellular genes near the site of integration of the retrovirus. This mechanism has been shown to contribute to tumorigenicity in animals (van Lohuizen, M. and Berns, A. (1990), Biochim. Biophys. Acta 1032: 213-235). These two observations have encouraged the development of Self-Inactivating-Vectors (SIN) in which retroviral promoters are functionally inactivated in the target cell (WO 94/29437). Further modifications of these vectors include the insertion of promoter gene cassettes

within the LTR region to create double copy vectors (WO 89/11539). However, in both these vectors the heterologous promoters inserted either in the body of the vector, or in the LTR region are directly linked to the therapeutic gene.

The previously described SIN vector mentioned above carrying a deleted 3'LTR (WO 94/29437) utilizes in addition a heterologous promoter such as that of Cytomegalovirus (CMV), instead of the retroviral 5'LTR promoter (U3-free 5'LTR) to drive expression of the vector construct in the packaging cell line. A heterologous polyadenylation signal is also included in the 3'LTR (WO 94/29437).

The object of the present invention is the construction of a safe retroviral vector, which harbours a cytochrome P450 gene as a therapeutic principle. This novel vector carries heterologous constitutive, inducible or tissue specific promoter and/or regulatory elements in the 3'LTR which, after infection become duplicated and translocated to the 5'LTR in the target cell. Thus in the infected cell the introduced promoter controls the expression of the cytochrome P450 gene, which is inserted into the body of the vector. This vector does not undergo self-inactivation - but instead promoter exchange, giving rise to the name ProCon vector for Promoter Conversion vectors. The principles and advantages of the ProCon system are described in more detail in WO 9607748.

Since Promoter Conversion does not result in Self-Inactivation, the retroviral vector will be transcriptionally active in the target cell. Additionally both LTR's will consist to a large extent of heterologous promoter/enhancer sequences in the target cell. This will reduce the likelihood of the integrated vector in the target cell being subject to the same inactivation over long periods as has been described for conventional vectors (Xu, L., Yee, J. K. et al., (1989), Virology 171: 331-341) and also will reduce the chance of recombination with endogenous retroviral sequences to generate potentially pathogenic replication competent virus, increasing the safety of the system.

According to the invention the 5'LTR of the retroviral vector construct is not modified, and expression of the viral vector in the packaging cells is driven by the normal retroviral U3 promoter. Normal retroviral polyadenylation is allowed, and no heterologous polyadenylation signals are included in the 3'LTR. This is important for the development of *in vivo* gene therapy strategies, since the normal physiological regulation of the virus, through the normal viral promoter, and possibly also involving the normal viral control of polyadenylation, will prevail over long periods *in vivo* whilst the packaging cells are producing recombinant virus.

To achieve the foregoing and other objects, the invention provides a retroviral vector undergoing promoter conversion comprising a 5' LTR region of the structure U3-R-U5; one or more coding sequences selected from the group of genes known as cytochrome P450 genes; and a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a heterologous promoter, followed by the R and U5 region.

Said promoter can either be constitutive as the Cytomegalovirus (CMV) immediate early promotor/enhancer, inducible such as by glucocorticoid hormones (eg the MMTV promoter) or target cell specific.

The target cell specific regulatory elements and promoters are selected from one or more elements of any gene but in this embodiment may be from promoters including carbonic anhydrase II and β -glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including carbonic anhydrase II and β -glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements of Whey Acidic Protein (WAP), Mouse Mammary Tumour Virus (MMTV), β -lactoglobulin and casein specific regulatory elements and promoters, pancreas specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic specific regulatory elements and promoters and MMTV specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland. Other promoters include for example the CD4, CD34, and IL2 promoters. Said regulatory elements and promoters regulate preferably the expression of said retroviral vector.

It appears that the region of the WAP promoter which is required for mediating the mammary gland specificity is a 320 bp XhoI/XbaI restriction fragment (-413 to -93) (Kolb, A. F., Günzburg, W. H., Albang, R., Brem, G., Erfle, V., and Salmons, B. (1995), *Biochem. Biophys. Res. Commun.* 217, 1045-1052). In addition certain experiments indicate that a 0.6 Kb PstI MMTV promoter fragment (Salmons, B., Groner, B., Calberg Baca, C. M., and Ponta, H. (1985), *Virology* 144: 101-114) may play a role in regulating the mammary gland specificity of expression displayed by MMTV (Kolb, A. F., Günzburg, W. H., Albang, R., Brem, G., Erfle, V., and Salmons, B. (1995), *Biochem. Biophys. Res. Commun.* 217, 1045-1052).

The LTR regions are selected from at least one element of the group consisting of LTRs of Murine Leukaemia Virus (MLV), Mouse Mammary Tumour Virus (MMTV), Murine Sarcoma Virus (MSV), Simian Immunodeficiency Virus (SIV), Human Immunodeficiency Virus (HIV), Human

T-cell Leukaemia Virus (HTLV), Feline Immunodeficiency Virus (FIV), Feline Leukaemia Virus (FELV), Bovine Leukaemia Virus (BLV) and Mason-Pfizer-Monkey virus (MPMV).

The retroviral vector is preferably based on either a LXS vector (Miller, A. D. and Rosman, G. J. (1989), *Biotechniques* 7: 980-990), pBAG (Price, J., Turner, D. et al., (1987), *Proc. Natl. Acad. Sci. USA* 84:156-160) or a hybrid of both.

The coding sequence of the therapeutic gene may be any cytochrome P450 gene but most preferably it is the rat cytochrome P450 form 2B1 defined by Fuji-Kuriyama, Y., Mizukami, Y., et al., (1982) *Proc. Natl. Acad. Sci. USA* 79:2793-2797.

In a further embodiment of the invention a retroviral vector system is provided comprising a retroviral vector as described above as a first component and a packaging cell line harbouring at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.

The packaging cell line is preferably selected from an element of the group consisting of Ψ -2, Ψ -Crypt, Ψ -AM, GP+E-86, PA317 and GP+envAM-12, or of any of these supertransfected with recombinant constructs allowing expression of surface proteins from other enveloped viruses.

The invention includes also mRNA resulting from a retroviral vector according to the invention.

In the packaging cell line the expression of the retroviral vector is regulated by the normal unselective retroviral promoter contained in the U3 region. However, as soon as the vector enters the target cell promoter conversion occurs, and the P450 gene is expressed from a tissue specific or inducible promoter of choice inserted into the ProCon vector. Not only can virtually any tissue specific promoter be included in the system, providing for the selective targeting of a wide variety of different cell types, but additionally, following the conversion event, the structure and properties of the retroviral vector no longer resembles that of a virus. This, of course, has extremely important consequences from a safety point of view.

These vector systems will be used to generate recombinant virus that can be used to infect tumor or normal cells either *in vitro* or *in vivo*.

Recombinant retroviruses which has been purified or concentrated may be preserved by first adding a sufficient amount of a formulation buffer to the media containing the recombinant

retrovirus, in order to form an aqueous suspension. The formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The recombinant retrovirus can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude recombinant retrovirus described above may be clarified by passing it through a filter, and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Northborough, MA). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated to remove excess media components and establish the recombinant retrovirus in a more desirable buffered solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified recombinant retrovirus is eluted. A sufficient amount of formulation buffer is added to this eluate to reach a desired final concentration of the constituents and to minimally dilute the recombinant retrovirus, and the aqueous suspension is then stored, preferably at -70°C or immediately dried. As noted above, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The crude recombinant retrovirus can also be purified by ion exchange column chromatography. In general, the crude recombinant retrovirus is clarified by passing it through a filter, and the filtrate loaded onto a column containing a highly sulfonated cellulose matrix. The recombinant retrovirus is eluted from the column in purified form by using a high salt buffer. The high salt buffer is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant retrovirus and the aqueous suspension is either dried immediately or stored, preferably at -70°C .

The aqueous suspension in crude or purified form can be dried by lyophilisation or evaporation at ambient temperature. Specifically, lyophilisation involves the steps of cooling the aqueous suspension below the glass transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilised retrovirus. Once lyophilised, the recombinant retrovirus is stable and may be stored at -20°C to 25°C , as discussed in more detail below.

Within the evaporative method, water is removed from the aqueous suspension at ambient

temperature by evaporation. Water can also be removed through spray drying.

The aqueous solutions used for formulation, as previously described, are composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the recombinant retrovirus upon freezing and lyophilization, or drying through evaporation.

The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilised or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin. The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilised state.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range desired, preferably between 7.0 and 7.8.

Aqueous solutions for the formulation of recombinant retroviruses are described in detail in WO-A2-96121014.

In addition, it is preferable that the aqueous solution contain a neutral salt which is used to adjust the final formulated recombinant retrovirus to an appropriate iso-osmotic salt concentration.

Lyophilized or dehydrated retroviruses may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted retrovirus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted retrovirus. Lyophilized or dehydrated recombinant retrovirus may be reconstituted with any convenient volume of water or the reconstituting agents that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

Recombinant retroviral particles may be administered to a wide variety of locations including, for example, into sites such as an organ or to a site of a tumor. Within other embodiments, the recombinant retrovirus may be administered orally, intravenously, buccal/sublingual, intraperitoneally, or subcutaneously. The daily dosage depends upon the exact mode of administration, form in which administered, the indication toward which the administration is directed, the subject involved and the body weight of the subject involved, and further the preference and experience of the physician in charge.

The routes of administration described herein may be accomplished simply by direct administration using a needle, catheter or related device. In particular, within certain embodiments of the invention, one or more dosages may be administered directly.

In one embodiment of the invention the packaging cells will be enclosed in capsules. For an effective treatment, the virus producing cells have to survive long periods in the target organ after implantation and virus must be produced during this period and released from the packaging cells. Thereby, the packaging cells producing the virus would, in effect, constitute a small virus producing factory, placed at the site of application. This will allow efficient delivery of the recombinant virus *in vivo*. Alternatively infected normal cells, either of human origin, or those originating from other species will be encapsulated and implanted, providing a small prodrug conversion factory that can be sited near or in the tumour mass.

The long term effectivity of this approach depends on (1) protection of the cells from the host immune system, which would normally eliminate virus producing or infected cells, especially if the cells are from a different species as is usually the case for retroviral vector producing cells and (2) survival of the cells *in situ* for extended periods, which may require vascularisation.

It has been found that the continuous production of a vector virus from implanted packaging cells can be achieved by the appropriate encapsulation, in microcapsules with semipermeable membranes, of the virus producing packaging cells before implantation. Additionally, it has been found that such capsules become well engrafted in the host, become vascularized, and do not elicit a host immune or inflammatory response. These findings, together with the semipermeability of the capsule membrane, permits long term retroviral vector delivery *in vivo*.

An encapsulation technology providing for the encapsulation of virus producing packaging cells, and of virus infected or normal cells in a cellulose based material has been developed. Using this technique up to 10^{10} , but preferably 10^5 - 10^7 cells are encapsulated in electrolyte complex (e.g. from alginate and polylysine or, more preferably, cellulose sulphate and polydimethyl-diallylammonium chloride) or other porous structures (such as polyamides, polysulphones). The resulting capsules can have a variable diameter between 0.01 and 5 mm, but preferably 0.1 and 1 mm. Consequently, capsules can be made to contain a variable number of cells. The capsule is semipermeable with pores that are large enough to allow viruses or prodrug molecules to pass through but small enough prevent cells of the immune system from accessing the cells thereby significantly reducing an immune response to these cells. The capsules and the encapsulated cells are cultivated in a normal cell culture medium (the nature of which depends on the cell line encapsulated) at standard conditions of humidity, temperature and CO_2 concentration.

After a suitable period in culture (normally not less than 1 hour and not exceeding 30 days), the cell containing capsules can be surgically implanted either directly, or by injection using a syringe into various areas.

At different times after the implantation of the encapsulated cells, the host can be treated with cyclophosphamide or ifosfamide either locally or systemically. Cells infected with the cytochrome P450 expressing virus will convert these prodrugs to the active metabolites which cause alkylation and cross-linkage of DNA. Also cells carrying and expressing the cytochrome P450 gene (such as encapsulated infected cells, or encapsulated packaging cells) will also catalyse this conversion. In one embodiment of this invention these encapsulated infected or packaging cells will be either slowly dividing cells, or cells that have been treated with mitomycin C, low doses of radiation, or other means to prevent cell replication, and thus to prevent the cells from being themselves affected by the cytotoxic effects of the prodrugs.

The following examples will illustrate the invention further. These examples are however in no way intended to limit the scope of the present invention as obvious modifications will be apparent, and still other modifications and substitutions will be apparent to anyone skilled in the art.

Example 1

This examples describes the construction of a retroviral expression vector for intratumoral infection which contains the gene for rat cytochrome P450 2B1.

Expression vector pLX2B1, shown in Figure 1, was constructed by ligation of fragments obtained from plasmid pLX125 and pSW1 (Kedzie, K. M., Escobar, G. Y., Grimm, S. W., He, Y. A., Pepperl, D. J., Regan, J. W., Stevens, J. C., and Halpert, J. R. (1991), J. Biol. Chem. 266(33): 2215-2). pLX125 was prepared as described in PCT/EP96/ 04447).

The plasmid pLX125 was linearized with HpaI and the resulting blunt ends dephosphorylated using calf intestine phosphatase. The DNA was purified by separation on a 1% agarose gel, excision and preparation using the Qiaquick protocol (Qiagen). After ethanol precipitation the DNA was resuspended in water.

The cloning vector pSW1 was digested with SmaI and HincII to yield two blunt ended fragments. The digestion mixture was separated on a 1% agarose gel. The shortest fragment (1,5kb) containing the rat cytochrome P450 2B1 cDNA (Fuji-Kuriyama, Y, Mizukami, Y. et al (1982), Proc. Natl. Acad. Sci. USA 79: 2793-2797) was excised and eluted using the Qiaquick DNA extraction protocol, ethanol precipitated and resuspended in water.

7,6 fMols of pLX125 and 24 fMols of the SmaI/HindII-fragment of pSW1 were mixed together and ligated for 3 days at 12°C using T4-ligase (Boehringer). The ligase was inactivated at 65°C for 10 min and the DNA butanol precipitated with a 10 fold volume of butanol. The precipitated DNA was resuspended in water and electroporated into DH10B-bacteria (Gibco). Ampicillin resistant colonies were selected, DNA prepared and test digested with SspBI/SalI, BamHI/SspBI. PvuI and BamHI. The final correct plasmid was designated pLX2B1 (see Fig 1).

Lipofection

One day before lipofection 3×10^6 retroviral packaging cells PA317 (Miller, A. D. and Buttimore, C. (1986), Mol. Cell. Biol. 6: 2895-2902) were seeded into 10 cm petri or culture dishes. On the day of infection 4 µg of pLX2B1 were mixed with 300µl serum free media. In parallel 45µl of Lipofectamine (Gibco BRL) was mixed with 300µl serum free media. The plasmid containing solution was added to the Lipofectamine-mix and incubated for 45min. After 35 min the cells were washed once with 6 ml serum free media. 2.4 ml of serum free media were added to the

lipofection-mix and the resulting 1ml was put onto the prepared cells. After 5 hours 3.5 ml Dulbecco's modified Eagles medium containing 20% FCS was added. The next day the cells were trypsinised and 1:20 diluted and seeded on a 100 mm dish. After 24h the media was replaced with medium containing the neomycin analogue G418. Cell populations were isolated and analysed for expression of cytochrome P450.

Supernatant from these cell populations was used to infect target CK cells. 1ml of virus containing supernatant from 5×10^6 cells was filtered through a $0.45\mu\text{m}$ filter and added to 1×10^6 target CK cells in the presence of $8\mu\text{g/ml}$ polybrene. After 4 hours ml of Dulbecco's Modified Eagles Medium with 10% Foetal Calf Serum was added.

Cells were trypsinized the next day, diluted and 24 hours later put in selection medium containing additionally $400\mu\text{g/ml}$ G418. After 2 weeks G418 resistant colonies were isolated and tested for cytochrome P450 2B1 activity. 2×10^4 cells were plated onto a 3 cm dish and exposed to concentration of ifosfamide varying between 0 and 5 mM. A higher sensitivity of the cytochrome P450 2B1 retrovirus infected cells was observed compared to control, non- infected cells.

Encapsulation

The retroviral vector producing packaging cells obtained are encapsulated as described in example 2 in WO 97/01357.

Implantation

The capsules obtained are introduced by "key hole" surgery near or in either transplanted or spontaneous tumours of BALB/c or GR mice. About six capsules of 1 mm diameter are inserted at each operation site. The site of surgery is closed by 1 suture. The mice are then treated with cyclophosphamide or ifosfamide locally, by direct intratumoral injection of $100\mu\text{l}$ of 20 mg/ml or systemic concentrations of 130 mg CPA/kg body weight i.p. and 40-60 mg IFO/kg body weight i.p. for up to a maximum of 10 weeks. During this period tumour size and macroscopic appearance is monitored daily. The mice are then sacrificed, the tissue containing the inserted capsules and tumour removed, and histological sections for light and electron microscopy prepared. These sections clearly show good engraftment of the capsules, vascularisation, and no evidence of the presence of lymphocytes indicative of a cellular immune response. These sections also show no sign of cell death or necrosis within the capsule. In contrast the tumour showed necrosis and macroscopically there was a clear reduction in size over the test period.

Example 2

This example describes the construction of a stable cell line which expresses rat cytochrome P450 2B1 constitutively.

Expression vector pc3/2B1 was constructed by ligation of fragments obtained from plasmid pcDNA3 (Invitrogen) and pSW1 (Kedzie, K. M., Escobar, G. Y., Grimm, S. W., He, Y. A., Pepperl, D. J., Regan, J. W., Stevens, J. C., and Halpert, J. R. (1991), *J. Biol. Chem.* 266(33): 2215-21).

The plasmid pcDNA3 was digested with XhoI/XbaI and the resulting sticky ended fragments dephosphorylated using calf intestine phosphatase. The DNA of the vector backbone was purified by separation on a 1% agarose gel, excision and preparation using the Qiaquick protocol (Qiagen). After ethanol precipitation the DNA was resuspended in water.

The cloning vector pSW1 was digested with XhoI and XbaI to yield two fragments. The digestion mixture was separated on a 1% agarose gel. The shortest fragment (1.5 kb) containing the rat cytochrome P450 2B1 cDNA (Fuji-Kuriyama, Y., Mizukami, Y. et al., (1982), *Proc. Natl. Acad. Sci. USA* 79: 2793-2797) was excised and eluted using the Qiaquick DNA extraction protocol, ethanol precipitated and resuspended in water.

8.3 fMols of the pcDNA3 backbone and 24.8 fMols of the XhoI/XbaI-fragment of pSW1 were mixed together and ligated for 1 day at 12°C using T4-ligase (Boehringer). The ligase was inactivated at 65°C for 10 min and the DNA butanol precipitated with a 10 fold volume of butanol. The precipitated DNA was resuspended in water and electroporated into DH10B-bacteria (Gibco). Ampicillin resistant colonies were selected, DNA prepared and test digested with EcoRI, BamHI, EcoRV and XhoI. The final correct plasmid was designated pc3/2B1.

Lipofection

Before the day of transfection 3×10^6 cat kidney cells were seeded into 100 mm dishes. On the day of transfection 4 µg of pc3/2B1 was mixed with 100 µl serum free media. In parallel 15 µl Lipofectamine was mixed with 100 µl serumfree media. The plasmid containing solution was added to the Lipofectamine-mix and incubated for 45 min. After 35 min the cells were washed once with 2 ml serum free media. 800 µl of serum free media were added to the lipofection-mix and the resulting 1 ml was put onto the prepared cells. After 6 hours 1 ml DMEM (Glutamax) with 10% FCS were added. The next day the cells were trypsinised and diluted by factor ten and seeded on a

100mm dish. After 24h the media was replaced against neomycin media. After 14 days neomycin resistant clones were isolated and tested for presence and activity of the vector.

Cytochrome P450 expressing cat kidney cells showed a tenfold higher sensitivity to ifosfamide or cyclophosphamide in comparison to wild type cells.

A bystander effect could be demonstrated on cat kidney wild type cells but also on pancreatic tumour derived Rin5 cells. This was shown by co-culturing the cytochrome P450 2B1 cat kidney cell clones with either non-producing cat kidney wild type cells or Rin5 rat pancreatic tumour derived cells and addition of ifosfamide. The non-producing CK and Rin cells are killed by the toxic metabolites of the ifosfamide which are produced and released by the cytochrome P450 2B1 cat kidney cells. Titration studies using one cytochrome P450 2B1 cat kidney cell clones showed that as little as 0.25mM ifosfamide causes specific toxic effects on these cells and 1-2mM results in the death of all cells. Cytochrome P450 2B1 cat kidney cell clones were shown by Polymerase Chain Reaction analysis to have acquired the P450 2B1 gene construct DNA. Further biochemical analysis of the clones using the enzymatic dealkylation of 7-pentoxoresorufin by cytochrome P450 2B1 revealed that the genetically modified cells are producing cytochrome P450 2B1.

Capsules containing these cells were produced as described in example 1 and implanted into mice near the tumour site. After treatment with cyclophosphamide or ifosfamide the efficacy of treatment was evaluated as described above.

A retroviral expression vector which contain the gene encoding rat-cytochrome P450-2B1 can also be prepared as described in example 4 below:

Example 3

The plasmid pLX125 was first partially digested with the restriction enzyme XhoI to yield a vector which is linearized at position 3547. This linear plasmid was further digested with the restriction enzyme SspBI to remove a short fragment within the polylinker of pLX125. In a preparative gel the correctly cut vector fragment appeared as the largest band. Using the Quiaex protocol, (Qiagen) the DNA in this band was eluted and purified from the gel.

To yield the rat cytochrome P450 2B1 gene cells of the rat hepatoma cell line HTC were lysed with solution D (4M guanidium thiocyanate, 25mM sodium citrate pH 7, 0.5%N-laurylsarcosine

sodium, 0.1M 2-mercaptoethanol) and total RNA extracted by adding 1/15 volume of 3M sodium acetate, in the same volume of water saturated phenol and 1/5 volume of chloroform/isoamylalcohol (49:1) were added and the whole mixture mixed vigorously. After 15 min on ice the extract was centrifuged 20min at 4°C at 10.000g. The RNA in the Aqueous phase was precipitated with one volume of isopropanol for 30 min at -20°C and centrifuged at 10.000g at 4°C. The pellet was washed in 70% ethanol and left at room temperature for 15 min. After 5 min centrifugation at 4°C and 10.000g the pellet was dried in a vacuum dryer and redissolved in 0.5% SDS solution.

The extracted RNA was reverse transcribed using the protocol for cDNA synthesis (Pharmacia). The resulting cDNA was used as template for a PCR. The primers were designed so that they contained a SspBI restriction site (underlined) in the left hand primer (5'-AAGCCTGTACACTGGAGAGCATGCAC-3') and a XhoI site (underlined) in the right hand primer (5'-CGATTACTCGAGACCTGGCTGCCTCA-3'). Both primers had additional bases at the 5'-end for higher efficiency of cleavage by the relevant restriction enzyme. The 1562 bp-product was digested with XhoI and SspBI to yield three fragments.

This longest fragment (1545 bp), containing the gene for cytochrome P450, was ligated into the XhoI/SspBI digested plasmid pLX125.

A stable cell line which expresses rat cytochrome P450 form 2B1 constitutively can also be prepared as described in example 5 below:

Example 4

To yield the mRNA from the rat cytochrome P450 form 2B1, a four week old female rat was sacrificed, the liver taken out and immediately frozen in liquid nitrogen. The frozen liver was put into sterilized filtered GTC-buffer (6M guanidium isothiocyanate, 5mM sodium citrate, 0,1M 2-mercaptoethanol, 0,5% sodium N-laurylsarcosyl) and homogenized at room temperature. For RNA separation the liver extract was put onto a cushion of caesiumchloride (5,7M Cesiumchloride, 0,1M EDTA) and centrifuged in a swing-out rotor at 20°C and 32.000rpm over night. After complete removing the supernatant the pelleted RNA was re-dissolved in ice cold 10mM Tris pH 7,5 and precipitated overnight at -20°C with 1/15 volume 3M sodium acetate and 2,5 volumes ethanol. The RNA was spinned down 40min at 8000rpm and 4°C and the dried pellet resuspended in sterile water.

The extracted RNA was reverse transcribed using the protocol for cDNA synthesis (Pharmacia). The resulting cDNA was used as a template for the following PCR. The primer were so designed

that they contained a EcoRI restriction site in the left hand primer (5'-CGTGC~~CGGAATTC~~GGCGGATTCAGCAT-3') and a EcoRV site in the right hand primer (5'-ATAACGGATATCACCTGGCTGCCTCA-3'). Both primers had additional bases at the 5'-end for higher efficiency of the cutting enzyme. The 1588 bp-amplificate was digested with EcoRI and EcoRV to yield three fragments.

This longest fragment (1572 bp), containing the gene for cytochrome P450 2B1, was ligated to the EcoRI/EcoRV digested plasmid pcDNA3 (Invitrogen).

Example 5

Construction of a ProCon vector containing the rat cytochrome P450 gene under transcriptional control of the WAP promoter.

The extracted RNA prepared in example 4 was reverse transcribed using the protocol for cDNA synthesis (Pharmacia). The resulting cDNA is used as a template for a PCR. The primers are designed so that they contain a BamHI restriction site (underlined) in the left hand primer e.g 5'-AAGCCGGATCCCTGGAGAGCATGCAC-3') and a BamHI site (underlined) in the right hand primer (e.g 5'-CGATTAGGATCCCTGCCTCA-3'). Both primers have additional bases at the 5'-end for higher efficiency of cleavage by the relevant restriction enzyme. The 1562 bp product is digested with BamHI and the fragment obtained containing the gene for cytochrome P450 is ligated into the BamHI digested plasmid pWAP.6 (see PCT application No. PCT/EP96/03922).

Claims:

1. A replication-defective retroviral vector carrying a cytochrome P450 gene under transcriptional control of target cell specific regulatory elements or promoters, or X-ray inducible promoters.
2. A replication-defective retroviral vector according to claim 1, wherein the vector comprises a 5' LTR region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence containing a target cell specific regulatory element or promoter, or an X-ray inducible promoter, followed by the R and U5 region, characterized in that at least one of the coding sequences codes for cytochrome P450.
3. A replication-defective retroviral vector according to claims 1 to 2, wherein the target cell specific regulatory element or promoter is selected from one or more elements of the group consisting of WAP, MMTV, b-lactoglobulin and casein specific regulatory elements and promoters, pancreas specific regulatory elements and promoters including carbonic anhydrase II and b-glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic specific regulatory elements and promoters and MMTV specific regulatory elements and promoters conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland.
4. A replication-defective retroviral vector according to claims 1 to 3, wherein said LTR regions are selected from at least one element of the group consisting of LTR's of MLV, MMTV, MSV, SIV, HIV, HTLV, FIV, FeLV, BLV and MPMV.
5. A replication-defective retroviral vector according to claims 1 to 4, wherein said retroviral vector is based on a BAG vector, or a pLXSN vector.
6. A replication-defective retroviral vector according to claim 5, wherein said retroviral vector is pLX2B1 prepared as described in example 1.
7. A replication-defective retroviral vector according to claim 5, wherein said retroviral vector is pc3/2B1 prepared as described in example 2.

8. A replication-defective retroviral vector according to claims 1 to 5, wherein the retroviral sequences involved in integration of the retrovirus are altered or at least partially deleted.
9. A replication-defective retroviral vector according to claim 1, wherein said regulatory elements or promoters are regulatable by transacting molecules.
10. A packaging cell line transfected with a replication-defective retroviral vector according to claims 1 to 9, said packaging cell line harbouring at least one retroviral or recombinant retroviral construct coding for the proteins required for said retroviral vector to be packaged.
11. A packaging cell line according to claim 10 wherein the packaging cell line is of rodent, canine, feline or human origin, and is histocompatible with human tissue.
12. A packaging cell line according to claim 10, where the packaging cell line is selected from the group consisting of psi-2, psi-Crypt, psi-AM, GP+E-86, PA317 and GP+envAM-12.
13. A recombinant retroviral particle produced by culturing a packaging cell line according to claims 10 to 12 under suitable conditions optionally followed by isolation of the recombinant retroviral particle produced.
14. A pharmaceutical composition comprising a recombinant retroviral particle according to claim 13.
15. A pharmaceutical composition comprising a packaging cell line according to claims 10 to 12.
16. A packaging cell line according to claims 10 to 12 which is encapsulated in a porous membrane which is permeable to the recombinant retroviral particles produced by said packaging cell line.
17. A packaging cell line according to claim 16 which is encapsulated in a complex formed from cellulose sulphate and polydimethyldiallylammonium.
18. A method for the ablation of tumour cells comprising administering to a subject in need thereof, a therapeutically effective amount of a recombinant retroviral particle according to claim

13, a packaging cell line according to-claims 10 to 12, or encapsulated packaging cells according to claims 16 to 17 and, either simultaneously or with a time span, a cancer prodrug which are activated by cytochrome P450.

19. A method according to claim 18 wherein the tumour cells are cells of breast tumours, or pancreatic tumours.

20. A method according to claim 18 wherein the cancer prodrug is cyclophosphamide, or ifosfamide.

21. A method according to claims 18 to 20 wherein the recombinant retroviral particle, the packaging cell line, or the encapsulated packaging cell line is administered by injection, or by implantation into the tumour, or at the site of the tumour and the cancer prodrug is administered systemically, or locally.

22. The use of a recombinant retroviral particle according to claim 13, a packaging cell line according to claims 10 to 12, or encapsulated packaging cells according to claims 16 to 17 for the preparation of a pharmaceutical composition useful for the ablation of tumour cells.

22. A retroviral provirus integrated in the human genome carrying a cytochrome P450 gene under transcriptional control of a target cell specific regulatory element or promoter, or an X-ray inducible promoter.

23. A human cell containing a cytochrome P450 gene under transcriptional control of a target cell specific regulatory element or promoter, or an X-ray inducible promoter.



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B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 97 09440 A (BAVARIAN NORDIC RESEARCH INSTITUTE; FORSCHUNGSZENTRUM FÜR UMWELT (DE);) 13 March 1997 see example 5	1-5, 8-23
X	WO 96 04789 A (GEN HOSPITAL CORPORATION; BOSTON UNIVERSITY; DANA FARBER CANCER INST) 22 February 1996	1, 2, 4, 8-23
Y	see page 38; example 5	3, 5
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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H.F. TIANO ET AL.: "Retroviral mediated expression of human cytochrome P450 2A6 in C3H/10T1/2 cells confer transformability by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK)" CARCINOGENESIS, vol. 14, no. 7, July 1993, pages 1421-1427, XP002040462 see figure 1	1,2,4, 8-13
Y	--- B. SALMONS ET AL: "Construction of retroviral vectors for targeted delivery and expression of therapeutic genes" LEUKEMIA, vol. 9, no. SUPPL. 01, June 1995, pages S53-S60, XP000575934 see figure 4	3,5
X	--- M.X. WEI ET AL.: "Experimental tumor therapy in mice using the cyclophosphamide-activating cytochrome P450 2B1 gene" HUMAN GENE THERAPY, vol. 5, no. 8, August 1994, pages 969-978, XP002040463 see the whole document -----	1,2,4, 8-23

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18-21
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 18-21 are directed to a method of treatment of the human or animal body, the search has nevertheless been carried out and was based on the alleged effects of the compositions.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/01585

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9709440 A	13-03-97	AU 6987696 A	27-03-97
WO 9604789 A	22-02-96	CA 2197677 A	22-02-96
		EP 0776161 A	04-06-97

Form PCT/ISA/210 (patent family annex) (July 1992)